ESTONIAN UNIVERSITY OF LIFE SCIENCES
Institute of Veterinary Medicine and Animal Sciences

Anja Kupiainen

BIOMARKERS FOR PREDICTING PROLONGED POSTPARTUM ANOVULATION IN DAIRY CATTLE

PIIMALEHMADEL POEGIMISJÄRGSELT PIKENENUD ANOVULATOORSET PERIOODI PROGNOOSIVAD BIOMARKERID

Final Thesis
Curriculum in Veterinary Medicine

Supervisor: Professor Andres Valdmann

Tartu 2021

Estonian University of Life Sciences
Kreutzwaldi 1, 51014, Tartu Estonia

Abstract of Final Thesis

Author: Anja Kupiainen

Curriculum: Veterinary Medicine

Title: Biomarkers for predicting prolonged postpartum anovulation in dairy cattle

Pages: 59	Figures: 2	Tables: 5	Appendixes: 1

Chair: Chair of Clinical Veterinary Medicine
Field of research and (CERC S) code: 3. Health, 3.2. Veterinary Medicine
B750 Veterinary medicine, surgery, physiology, pathology, clinical studies
Supervisor: Andres Valdmann
Place and year: Tartu 2021

Negative energy balance and inflammation have negative effect on resumption of ovarian activity in dairy cattle. The study aim was to establish a set of biomarkers allowing the prediction of cows with risk of developing prolonged postpartum anovulation (PPA). Previously collected data from multiparous Holstein cows (n=118) was analysed. Blood samples were collected at three time points (-2wk, +1wk and +3wk in relation to calving) and body condition score (BCS) was measured before and after calving (-2wk and +3wk). Cows were grouped into two groups (normal and PPA group) based on time of their first ovulation postpartum (PPA: milk progesterone >5 ng/ml at ≥ 50 days postpartum). Metabolites and hormones were analysed by autoanalyzer or ELISA. Significant variables and their thresholds were determined by ROC curve analysis. Variables with AUC >0.6 ($P<0.05$) were submitted to a multivariate logistic regression model with forward stepwise algorithm. PPA prevalence was 36.4%. Insulin-like growth factor-1 (IGF-1), non-esterified fatty acids, ß-hydroxybutyrate (BHB), insulin, serum amyloid A (SAA), haptoglobin, ceruloplasmin, albumin, aspartate aminotransferase and creatine kinase (CK) had AUC >0.6 ($P<0.05$) in predicting PPA on at least one of the three time points. In the multivariate logistic regression model plasma IGF-1, BHB, SAA and CK provided a set of biomarkers for predicting development of PPA with good accuracy. The multivariate logistic regression model discriminating cows with and without PPA generated area under the ROC curve of 0.87 (95% CI = 0.80 - 0.93; $P<0.001$). Inclusion of BCS did not improve the AUC of the model.

Keywords: prolonged anovulation, inflammation, negative energy balance, biomarkers

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Eesti Maaülikool Kreutzwaldi 1, 51014, Tartu		Lõputöö lühikokkuvõte	
Autor: Anja Kupiainen		Õppekava: Veterinaarmeditsiin	
Pealkiri: Piimalehmadel poegimisjärgselt pikenenud anovulatoorset perioodi prognoosivad biomarkerid			
Lehekülgi: 59	Jooniseid: 2	Tabeleid: 5	Lisasid: 1
Õppetool: Kliinilise veterinaarmeditsiini õppetool ETIS-e teadusvaldkond ja CERC S-i kood: 3. Terviseuuringud, 3.2 veterinaarmeditsiin B750 Veterinaarmeditsiin, kirurgia, füsioloogia, patoloogia, kliinilised uuringud Juhendaja: Andres Valdmann Kaitsmiskoht ja -aasta: Tartu 2021			

Negatiivne energiabilanss ja põletik mõjutavad negatiivselt piimalehmade munasarjade aktiivsuse taastumist. Uuringu eesmärk oli selgitada, millised vereplasma biomarkerid võimaldavad prognoosida lehmi, kellel on risk poegimisjärgselt pikenenud anovulatoorse perioodi (PAP) tekkeks. Töös analüüsiti varem kogutud andmeid. Vereproovid võeti 118 holsteini tõugu korduvpoeginud lehmalt kolmel erineval ajal (2 nädalat enne ning 1 ja 3 nädalat pärast poegimist). Kahel korral (2 nädalat enne ja 3 nädalat pärast poegimist) määrati lehmade kehakonditsiooni skoor (KKS). Esimese ovulatsiooni aeg määrati piima progesterooni (P4) profiilide abil. Kui P4 tõus >5 ng/ml esines enne 50. poegimisjärgset päeva, siis klassifitseeriti loom normaalse poegimisjärgse ovulatsiooni ajaga lehmade rühma. Kui P4 tõusuks >5 ng/ml kulus ≥50 päeva, siis klassifitseeriti loom PAP rühma. Metaboliidid ja hormoonid analüüsiti autoanalüsaatoriga või ELISA meetodil. Iga muutuja ja iga proovivõtu aja jaoks määrati ROC kõvera analüüsiga optimaalne klassifitseerimise kriteerium. Muutujad kõveraaluse pinnaga (AUC) vähemalt 0,6 ($P<0,05$) analüüsiti logistilise regressioonanalüüsiga. PAP esinemissagedus oli 36,4%. Insuliinisarnane kasvufaktor-1 (IGF-1), esterifitseerimata rasvhapped, ß-hüdroksübutüraat (BHB), insuliin, seerumi amüloid A (SAA), haptoglobiin, tseruloplasmiin, albumiin, aspartaadi aminotransferaas ja kreatiini kinaas (CK) omasid PAP prognoosimisel AUC >0,6 vähemalt ühes kolmest proovi võtmise ajapunktis. Mitmeparameetrilise logistilise regressioonanalüüsi mudelis olid olulisteks PAP prognoosivateks metaboliitideks 2 nädalat enne poegimist IGF-1 ja SAA, 1 nädal pärast poegimist CK ja 3 nädalat pärast poegimist BHB ja SAA. Mitmeparameetrilise logistilise regressioonanalüüsi mudeli AUC oli 0,87 (95% CI = 0,80 - 0,93; $P< 0,001$). KKS lisamine mudelisse ei parandanud mudeli prognostilist täpsust. Töö tulemusena leiti metaboliitide komplekt, mis võimaldab prognoosida PAP hea täpsusega.

Märksõnad: pikaajaline anovulatsioon, põletik, negatiivne energiabilanss, biomarkerid

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LIST OF ABBREVIATIONS

Alb	albumin
ANOVA	analysis of variance
APP	acute phase protein
AST	aspartate aminotransferase
AUC	area under curve
BCS	body condition score
BHB	beta-hydroxybutyrate
CK	creatine kinase
CL	corpus luteum
Cp	ceruloplasmin
CYTO	cytological endometritis
DIM	days in milk

ELISA	enzyme-linked immunosorbent assay
FSH	follicle stimulating-hormone
GH	growth hormone
GnRH	gonadotropin releasing-hormone
Hp	haptoglobin
IGF-1	insulin-like growth factor-1
IL-1β	interleukin 1β
IL-18	interleukin 18
LH	luteinizing hormone
LPS	lipopolysaccharide
NEB	negative energy balance
NEFA	non-esterified fatty acids
OR	odds ratio
PAMP	pathogen-associated molecular pattern
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PPA	prolonged postpartum anovulation
P4	progesterone
PRR	pattern recognition receptor
r	correlation coefficient
ROC	receiver operating characteristic
SAA	serum amyloid A
Se	sensitivity
Sp	specificity
TLR	toll-like receptor
TNFα	tumour necrosis factor α

INTRODUCTION

Dairy cattle's ability to regain the resumption of postpartum ovarian cyclicity straight after the voluntary waiting period is essential for maintaining regular calving interval and for the herd profitability. Modern dairy cattle encounter many challenges which have negative effect on fertility and normal ovarian function. Prolonged postpartum anovulation (PPA) period in dairy cattle increases the calving interval and at the same time has negative economic effect (Mwaanga and Janowski, 2000). It is generally agreed that cows who resume their postpartum ovarian activity and continue to cycle at regular intervals by the day 50 postpartum, are expressing normal ovarian function (Opsomer *et al.*, 1998).

There are multiple risk factors which can lead to PPA period in dairy cattle. Energy balance changes, body condition score (BCS) at calving, dry matter intake and health disorders are factors inhibiting the resumption of postpartum cyclicity (Crowe *et al.*, 2014). Common problem in dairy cattle in early postpartum period is negative energy balance (NEB), which occurs when energy intake does not meet the energy demands of maintenance and production (Walsh *et al.*, 2007). Non-esterified fatty acids (NEFA) and beta-hydroxy butyrate (BHB) are metabolites associated with NEB (Ospina *et al.*, 2010). Insulin and insulin-like growth factor (IGF-1) are metabolic hormones, and both are potential markers for intermediating the NEB effect on reproduction function (Llewellyn *et al.*, 2007).

Diseases and inflammations during periparturient period impair the reproductive performance of dairy cattle (Ribeiro *et al.*, 2016). Positive acute phase proteins (APPs) like serum amyloid A (SAA), haptoglobin (Hp) and ceruloplasmin (Cp) are early markers of inflammation (Kaya *et al.*, 2016). Increased levels of these three APPs are found e.g., in endometritis and their concentration correlates the severity of the disease (Kaya *et al*., 2016). Opposite to positive APPs, albumin (Alb) is negative APP, and its concentration decreases in case of inflammation or infectious disease (Eckersall, 2008. P.135). Other biomarkers

associated with inflammation are aspartate aminotransferase (AST) and creatine kinase (CK) (Sattler and Fürll, 2004; Kaya *et al.*, 2016).

According to U.S. Food & Drug Administration (FDA) biomarker is defined as characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic intervention. As inflammation and NEB negatively affect the postpartum resumption of ovarian cyclicity, monitoring biomarkers related to these conditions would possibly improve the chance of decreasing number of cows experiencing PPA. There are several biomarkers which are associated with prolonged anovulation, but the best set of early biomarkers allowing the prediction of cows with PPA need to be established.

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1. LITERATURE REVIEW

1.1. Normal physiology of ovarian function in cows

After pregnancy and parturition, uterus have to involute back to its pre-gravid size and resume the ovarian cyclicity. After parturition follicular stimulating hormone (FSH) starts to rise in three to five days and it takes seven to ten days for follicles to restart their growth (Crowe *et al.*, 2014). Usually, the first ovulation postpartum is silent, which is followed by short ovarian cycle (Crowe *et al.*, 2014).

Dairy cows are non-seasonal polyestrus animals, meaning that they undergo estrus cycle throughout the year. Estrus cycle in the normal non-pregnant cow is 21 days, but it can range individually from 18 to 24 days. During estrus cycle ovaries go through follicular and luteal phase. Follicular phase is further subdivided into proestrus and estrus and luteal phase into metestrus and diestrus (Sjaastad *et al.*, 2010, P.704).

As in other female mammals, gonadal activity in dairy cows is regulated by the hypothalamus-anterior pituitary-ovary axis (Sjaastad *et al.*, 2010, P.233-234). Hypothalamus releases gonadotropin releasing hormone (GnRH) in pulsatile pattern and this regulates the release of FSH and luteinizing hormone (LH) from the anterior pituitary (Sjaastad *et al.*, 2010, P.233-234). Ovarian function is stimulated by these two hormones, FSH and LH.

Ovarian follicular growth is stimulated by the FSH (Sjaastad *et al.*, 2010, P.709). FSH binds to the granulosa cell receptors, which leads to the proliferation and growth of these cells.

Theca cells are stimulated by LH which leads to production of testosterone. This testosterone is converted into estrogen by the granulosa cells under the influence of FSH. Along with producing estrogen, growing follicles produce inhibin, which in the anterior pituitary inhibits FSH secretion (Sjaastad *et al.,* 2010, P.709).

There are three critical points during the final stage of follicular growth: emergence (~4mm), deviation (~9mm) and ovulation (~10-20mm) (Wiltbank *et al*., 2002). Follicular emergence occurs at the peak of the FSH surge, and it is followed by follicular deviation when level of FSH is decreasing. At this point happens the dominant follicle selection. FSH inhibitors inhibin and estradiol are secreted by the follicles; all size follicles secrete inhibin, but estradiol level is increasing after selection of dominant follicle. Together these two FSH inhibitors have strong decreasing effect on FSH. After follicular deviation, LH has important role in follicular growth. Growing follicle produce large amount of estradiol. When certain level of estradiol is reached, it induces LH surge and ovulation of the dominant follicle within 24-32h (Wiltbank *et al*., 2002).

Ovulation is followed by formation of corpus luteum (CL) (Sjaastad *et al.,* 2010. P.712-713). Growing CL secrets progesterone (P4) which has vital role in preparing uterus for the initiation and maintenance of pregnancy. In both pregnant and non-pregnant animal pulsatile LH from anterior pituitary is needed for the maintenance of the CL. If fertilization does not occur, CL starts to regress and P4 level decreases in the second half of the luteal phase. Increased level of P4 secreted by CL triggers the timing of $PGF_{2\alpha}$ secretion. High concentration of circulating P4 leads to decreased level of P4 receptors in endometrium, which triggers $PGF_{2\alpha}$ secretion (Sjaastad *et al.*, 2010. P.712-713; Wiltbank *et al.*, 2002).

In dairy cows, group of small follicles develops synchronously during estrus cycle and this may occur two to four times (Wiltbank *et al.*, 2002). This wave-like development of follicles is referred as follicular waves. During follicular wave, biggest developing follicle becomes the dominant follicle and other follicles degenerate and go into atresia. Increase in FSH is essential for initiation of follicular wave. First dominant follicle is non-functional due to presence of functional CL and high P4 level. Second or sometimes third wave dominant

follicle continues to ovulate, because it is functional at the time when CL is regressing (Wiltbank *et al.*, 2002).

1.2. Prolonged postpartum anovulation

Dairy cow should be able to produce a calf per year to be productive and profitable to the farmer. With modern high producing dairy cattle this is increasing challenge due to the PPA. It is generally agreed that cows who resume their postpartum ovarian activity and continue to cycle at regular intervals by the day 50 postpartum, are expressing normal ovarian function (Opsomer *et al.*, 1998). Cows, which resume their ovarian function after day 50 postpartum are experiencing PPA period (Opsomer *et al.*, 1998).

1.2.1. Prevalence

Prevalence of the PPA in dairy cattle varies between herds and study groups. Reported prevalence values are e.g., 19% (Walsh *et al*., 2007), 23% (Moreira *et al*., 2001), 24.1% (Santos *et al*., 2009), 26.2% (Dubuc *et al*., 2012), and 35.2% (Dubuc *et al*., 2017). Differences between results can be explained by the variable level of herd management and nutritional status of individuals at farm level. Dubuc *et al*. (2017) suggest an alarm level of ≥21% of prolonged anovulation in herds.

1.3. Pathophysiology of prolonged postpartum anovulation

1.3.1. Types of anovulatory conditions

There has been distinguished four different types of anovulatory conditions based on the physiology of ovarian follicular and luteal dynamics.

In type I there is growth of follicles, but no deviation (Peter *et al.*, 2009). It is suggested that this condition is linked to undernutrition and energy deficit, which leads to nonsufficient LH concentrations in the body to support follicular growth. Decreased frequency in LH pulses may be due to increased negative feedback from estradiol or decreased GnRH neuronal activity (Peter *et al.*, 2009).

In type II there is follicular growth and deviation, but it is followed by follicular atresia or regression (Peter *et al.*, 2009). Contributing factor for the regression is insufficient estradiol production or failure of positive feedback system leading to low LH pulse frequency (Peter *et al.*, 2009).

In type III there is growth, deviation, and dominant follicle, but instead of ovulation there is persistent follicular structure (Peter *et al.*, 2009). Reason for it can be lack of sensitivity of hypothalamus for the positive feedback from estradiol. Other reason is due to metabolic hormones like IGF-1 and insulin altering the follicle ability to respond gonadotropins (Beam and Butler, 1999; Peter *et al.*, 2009).

In type IV follicle ovulate normally but CL does not regress normal, and the luteal phase is prolonged (Peter *et al.*, 2009). Variable factors can increase the risk of prolonged luteal phase e.g., parity, dystocia, postpartum complications, uterine infections, and heat stress (Opsomer *et al.*, 2000; Ranasinghe *et al.*, 2011; Peter *et al.*, 2009).

1.3.2. Cysts

Ovarian cysts are one reason in dairy cattle for the PPA and fertility problems. Cysts can be classified as follicular cysts or luteal cysts (Youngquist and Threlfall, 2008. P. 380-381). Follicular cysts have multiple layers of granulosa cells which secret estradiol. Luteal cysts

wall contains theca cells, and its granulosa cells can be luteinized. According to general understanding luteal cysts develop from follicular cysts when granulosa and theca cells luteinize over time. Luteal cysts secrete P4, and the concentration can be highly variable (Youngquist and Threlfall, 2008, P. 380-381). Brodzki *et al.* (2019) clearly demonstrated in their study the difference in circulating levels of sex steroid hormones 17β-estradiol and P4 based on the estrus phase and the type of cysts. Cows with follicular cysts or in the follicular phase of the cycle had high level of 17β-estradiol and low level of P4. High P4 level and low 17β-estradiol level was observed in cows with luteal cysts and during the luteal phase of the cycle.

Ovarian follicular cysts develop when one or more follicles fail to ovulate but maintain growth and steroidogenesis (Braw-Tal *et al.*, 2009). Pathology behind formation of ovarian follicular cysts is a failure of the hypothalamus induce the preovulatory surge of LH in response to estradiol. Metabolic hormones insulin and IGF-1 have essential role in the follicular growth before ovulation. Braw-Tal *et al*. (2009) suggest that abnormal level of these two hormones will result follicular dysfunction, which can lead to either follicular regression or cyst formation.

1.3.3. Bacteria, bacterial products, and cytokines

In bacterial diseases or infections bacteria or their products activate pattern recognition receptors e.g., toll-like receptors (TLR) or other pattern recognition receptors (PRRs) according to Gilbert (2019). These receptors respond to pathogen-associated molecular patterns (PAMPs) which are found in the cell walls of bacteria. Lipopolysaccharides (LPS) are typical PAMPs in the gram-negative bacteria e.g., E. coli (Gilbert, 2019). Bacterial products and endotoxins can directly reach ovaries via general circulation even from distant sites. Because ovarian follicular cells have also TLRs, bacterial products and endotoxins can directly impair their function. Activation of TLRs or other PRRs triggers the production of proinflammatory cytokines like interleukin 1β (IL-1β), interleukin 18 (IL-18) and tumor

necrosis factor α (TNFα). Like bacterial products, cytokines can reach ovaries via general circulation and can have direct effect on uterus and conceptus (Gilbert, 2019).

Herath *et al*. (2007) found out that LPS suppress oestradiol production in dominant follicles and recruited follicles. This applied to animals with uterine disease and LPS was detected in their follicular fluid and in vitro conditions when granulosa cells were exposed to LPS. LPS can directly affect granulosa cells because they express specific receptors for LPS complexes e.g., TLR4, CD14 and MD-2 mRNA transcripts.

Battagnia *et al.* (2000) studied endocrine changes of follicular phase in ewes caused by endotoxin. Their findings demonstrated interruptions in preovulatory estradiol rise caused by endotoxins, which led to delaying or blocking of the LH/FSH surge in all ewes. There can be more than one mechanism behind this and Battagnia *et al.* (2000) suggest three different mechanisms: 1) endotoxin can act at a neuroendocrine level, potently suppressing pulsatile LH release after P4 withdrawal, 2) endotoxin may also exert suppressive effects at the ovarian level, impairing follicular development and/or inhibiting estradiol secretion in response to gonadotropic stimulation, 3) endotoxin may compromise the LH surge system.

1.4. Risk factors for prolonged postpartum anovulation

There are many factors which influence the risk of PPA in dairy cattle. NEB and inflammation around periparturient period are most prevalent risk factors, but there are multiple other factors which present a risk for postpartum anovulation. According to Crowe *et al.* (2014) the main factors inhibiting the resumption of postpartum cyclicity were energy balance changes, BCS at calving, dry matter intake and health disorders.

1.4.1. Age

When comparing primiparous and multiparous cows, primiparous cows are more likely to express PPA period than multiparous cows (Plozza *et al*., 2016; Santos *et al*., 2009). Reason for this is due to the different energy requirements between these two groups. Primiparous cows not only need energy for their milk production, but also for their own growth. Primiparous cows have been shown to have higher circulating NEFA and BHB concentrations than multiparous cows in early lactation, which can affect the time of first ovulation (Meikle *et al*., 2004).

Dubuc *et al*. (2012) argue that cows of third parity or greater had lower probability of early ovulation than cows of first and second parity. Similar results were concluded by Lomander *et al.* (2012), saying that first calvers had less variation in plasma concentrations of NEFA and BHB compared with older cows. One explanation for this could be that cows of different parity may have different capacity to adapt to metabolic stress.

Circulating IGF-1 concentration gradually decline with age. Kerr *et al.* (1991) argues that young cows require higher circulating IGF-1 levels to support their growth until physical maturity is reached. In their study Gobikrushanth *et al.* (2018) observed 2-fold higher circulating IGF-1 concentrations with primiparous cows compared to multiparous cows.

1.4.2. Calving season

Even though cows are non- seasonal breeders, it has been shown that calving season influences the postpartum resumption of ovarian cyclicity. Cows calving in the spring and winter have greater risk to experience PPA than cows calving in the summer and fall (Walsh *et al*., 2007; Santos *et al*., 2009).

1.4.3. Nutrition

Relationship between early postpartum metabolic status and the postpartum resumption of ovarian activity have been investigated and the results are showing clear connection between them. There is also some evidence that energy status already during the dry period can have effect on postpartum resumption of ovarian activity. Castro *et al*. (2012) observed that dry period energy status is closely associated with BCS and resumption of ovarian activity postpartum investigating the plasma glucose, insulin, IGF-1, triiodothyronine and thyroxine levels and energy balance in their study.

In dairy cattle dietary restrictions induce catabolic environment which may be contributing factor for PPA. In such a case there is reductions in estradiol synthesis, granulosa cells responsiveness to FSH and IGF-1 signals is decreased and theca cells responsiveness to LH is also diminished (Walsh *et al*., 2012).

1.4.4. Energy balance

For dairy cows peripartum period is challenging time regarding energy balance. There is increasing energy requirements due to the growing fetus and starting of the lactation, and at the same dry matter intake is usually decreasing. NEB state occurs when energy demand for maintenance and production are higher than energy intake. NEB can lead to ketosis, which can increase the risk for PPA (Spicer *et al*., 1990; Walsh *et al*., 2007).

In NEB body fat stores are mobilized into the bloodstream in the form of NEFA and contribute to overall energy requirements during early lactation. The liver removes a large portion of the NEFA circulating in the bloodstream by metabolizing NEFA into ketone bodies, such as BHB, or re-esterifying them into triglycerides (Walsh *et al*., 2007). Dairy cattle with severe NEB have higher circulating NEFA and BHB concentrations compared to cows with mild NEB (Llewellyn *et al.*, 2007). NEB is a risk factor for the delayed resumption of postpartum cyclicity. If serum NEFA concentration is increasing during the prepartum

and postpartum period, at the same time increase the risk of delayed resumption of postpartum cyclicity (Jeong *et al.*, 2015).

Postpartum NEB affects the resumption of ovarian activity and fertility. According to Spicer *et al.* (1990), serum P4 concentration and weekly average energy balance are positively correlated. They argued that NEB has adverse effects on luteal function. They observed that cows with positive energy balance presented significantly higher P4 concentrations during first and second estrus cycle compared to cows with NEB. Low levels of serum IGF-1 concentrations are found in cows with NEB. Llewellyn *et al.* (2007) reported significantly lower levels of IGF-1 and insulin in cows with severe NEB compared to cows with mild NEB.

1.4.5. Body condition score

BCS reflects the nutritional state of the cow and it is considered as subjective method to determine the body fat reserves. Lower BCS at the time of calving and decreasing BCS in the early lactation period has negative effect on resumption of ovarian function and reproductive success (Pryce *et al*., 2001; Santos *et al*., 2009; Plozza *et al.*, 2016).

1.4.6. High milk yield

In dairy cattle milk yields effect on fertility has been studied widely and the results are controversial. Some researchers suggest high milk yield having negative effect on fertility. Lopez *et al.* (2004) identified a relationship between level of milk production at the time of estrous expression and the duration of estrus. Higher producing cows had lower estradiol concentrations one day before estrus and on the day of estrus compared to lower producing cows.

High producing dairy cows are more likely to experience NEB in the early postpartum lactation period. As mentioned earlier, NEB has negative effect on resumption of ovarian activity and fertility. Rearte *et al.* (2018) concluded in their study that the extent of the relationship between milk yield and reproductive performance is low and it is depended on the level of herd production. They proposed that the increased energy demands to support milk yield in early postpartum period is not the reason for NEB in high producing dairy cattle. Instead, the effect of energy balance on fertility is related to the amount of net energy consumed rather than with the amount of net energy secreted into milk.

1.4.7. Suckling

Suckling is one contributing factor for PPA, because suckling suppresses pulsatile LH secretion which determines whether the dominant follicle ovulate or not (Stagg *et al*., 1998). Generally, this not a problem in dairy cattle whose calves are separated from the dam soon after birth. Suckling is a major factor affecting the PPA in beef cattle and can be problem in organic dairy farms if calves are kept with their dams after birth.

1.4.8. Calving related condition: dystocia and twins

Dystocia is stressful situation for the cow and generally the prevalence is internationally <5% (Mee, 2008). Dobson *et al.* (2001) analysed the effects of stress to fertility. Difficult birth or caesarean section is acute stressor, and it has negative effect on fertility. Acute or chronic stressors disrupt the correct functioning of the hypothalamus-pituitary-ovarian axis. There is delayed uterine involution, abnormal ovarian cyclicity and prolonged next pregnancy interval.

Gaafar *et al.* (2010) studied the effects of dystocia on reproductive performance of Friesian cows and reported dystocia having negative effect on the reproductive performance.

Dystocia leads to increased service interval, service period, days open and calving interval. Dystocia increases the odds of developing metritis, retained placenta and vaginal discharge (Dubuc *et al.* 2010; Hossein-Zadeh and Ardalan, 2011).

Causes of dystocia are variable and it is difficult to find methods to reduce its occurrence. Twins are one reason for dystocia. Hossein-Zadeh (2010) describes significantly greater dystocia incidences in twin-births compared to single-births. Dystocia in twin-births is most likely result of abnormal presentation of the head and/or legs for one or both twin fetuses at parturition. Twin-births are risk factors for retained placenta (Hossein-Zadeh and Ardalan, 2011).

1.4.9. Diseases

In postpartum dairy cows, diseases have detrimental effect on fertility regardless the onset of the disease, whether the disease occurs prior parturition or in the early postpartum period (Ribeiro *et al.*, 2016). According to Gilbert (2019), common diseases for dairy cows affecting uterine health are retained foetal membranes, metritis, and endometritis. Common non-uterine diseases are ketosis, mastitis, displaced abomasum, clinical hypocalcaemia, and lameness. PPA, reduced conception rate and increased pregnancy wastage are consequences of presence of diseases.

In high producing dairy cows clinical and subclinical endometritis are common diseases and they delay the onset of postpartum ovarian cyclicity and extend luteal phase (Sheldon *et al*., 2009). Long luteal phase may be explained by the change in endometrial prostaglandin production from PGF to PGE. Association between uterine infection and PPA is due to bacteria and their products. Circulating bacteria can impair the function of the hypothalamus and pituitary, and directly disturb steroidogenesis in granulosa cells in ovaries. After ovulation, the corpus luteum secretes less P4 in diseased than in normal animals (Sheldon *et al.*, 2009).

In their study, Vieira-Neto *et al.* (2014) observed that both PPA and CYTO have a similar negative effect on reproductive performance. If these two conditions are combined, then their effect is additive. This effect was observed both in confinement dairy cows as well as in a grazing dairy cows.

1.4.10. Inflammation

Inflammatory status of the cow affects the postpartum resumption of ovarian activity. Sina *et al.* (2018) describe association between ovarian activity and inflammatory status of the cow. Uterine inflammation affects negatively on postpartum ovarian activity, and this was evident in cows with uterine inflammation. Cows with uterine inflammation had smaller first dominant follicle which secrete less estradiol 17β compared to healthy cows.

Results of the Sina *et al.* (2018) study showed an association between inflammatory status and different pattern of luteal activity, ovulation, and reproductive performance in early lactating Holstein dairy cows. The effect of inflammation activation is decreased luteal size, abnormal growth of largest follicle, declined estradiol and P4 concentrations, delayed ovulation, shortened or extended luteal phase after ovulation, increased time to first insemination, decreased conception rates and increased open days.

1.5. Markers of inflammation

1.5.1. Haptoglobin

Hp is glycoprotein and in ruminants it is important APP. Circulating level of Hp is below 2mg/dl (20mg/L), but in case of inflammation can increase up to 200mg/dl within couple of days of infection (Eckersall, 2008. P. 137-138). In cattle Hp is indicative marker of inflammation in diseases like mastitis, endometritis, and peritonitis. Increased levels of Hp

are also found in cases of fatty liver disease, at parturition, and during starvation (Eckersall, 2008. P. 137-138).

Hp levels are significantly higher in cows with endometritis than in healthy cows and the concentration correlates with the severity of endometritis (Kaya *et al*., 2016). Dubuc *et al.* (2012) showed that elevated Hp concentration during the postpartum period was associated with a longer period of postpartum anovulation. There are indications that Hp concentrations in the blood could be used as predictive biomarker for inflammation. According to Huzzey *et al.* (2009) acute phase inflammatory response precedes clinical metritis. Their study showed that postpartum cows on 3 days in milk (DIM) with Hp concentration ≥ 1 g/L were 6.7 times more likely to develop severe or mild metritis.

1.5.2. Serum amyloid A

SAA is a small protein, which is found in association with high density lipoprotein (HDL) in serum. In cattle SAA is inflammatory marker, which increase more in acute inflammations than in chronic conditions and it is considered as acute phase protein (Eckersall, 2008. P.138).

Similarly, to Hp, levels of SAA are significantly higher in cows with endometritis than in healthy cows and the concentration of this biomarker correlates with the severity of endometritis (Kaya *et al*., 2016).

1.5.3. Albumin

Alb is major serum protein being 35-50% of the total serum proteins. Alb concentrations decrease gradually in inflammatory and infectious diseases and it is referred as negative acute phase protein (Eckersall, 2008. P.132-135).

Krause *et al.* (2014) suggest that the serum Alb concentration could be an important biomarker of both the severity of uterine disease and the ovulatory potential in early postpartum lactating dairy cows. In their study, cows which showed no sign of postpartum endometritis had higher serum Alb concentrations and they resumed ovarian activity earlier during the postpartum period compared to cows with disease status.

Monitoring alb concentrations in postpartum dairy cows may be a relevant tool to monitor herd health status. Ruprechter *et al.* (2018) reported association between Alb concentrations measured two weeks before parturition with postpartum health status. In multiparous cows' Alb concentrations were predictive for metritis and retained placenta at week −2 and for retained placenta at week −1 in relation to calving.

1.5.4. Ceruloplasmin

Cp is a plasma α-2 glycoprotein and in the cattle, it is one of the APPs (Cerone *et al.*, 2000). It has important role in copper transport in the blood stream and in iron metabolism. Copper acts on different enzymes in the antioxidant system and Cp has important role in mediating the transport of copper (Cerone *et al*., 2000). In inflammatory conditions the need for Cp increase. If serum Cp levels fall, it leads to decreased antimicrobial activity and phagocytosis (Cerone *et al.*, 2000).

According to Kaya *et al.* (2016), there is significant correlation between serum Cp levels and Hp and SAA levels. They concluded that Cp levels can be used in the diagnosis of endometritis as an alternative to Hp and SAA levels.

1.6. Markers of negative energy balance

1.6.1. Non-esterified fatty acids and beta-hydroxy butyrate

Serum concentrations of NEFA and BHB can be used as energy status markers. During NEB body fat stores are mobilized into the bloodstream in the form of NEFA. The liver removes a large portion of the NEFA circulating in the bloodstream by metabolizing NEFA into ketone bodies, such as BHB or re-esterifying them into triglycerides (Herdt, 2000).
PPA is associated with greater serum NEFA and BHB concentrations (Dubuc *et al*., 2012). Screening of serum NEFA and BHB concentrations could help to identify cows at greater risk of PPA and possibly to give them preventive therapy. Hyperketonemia during the first week of postpartum is important risk factor for displaced abomasum, clinical ketosis, and metritis according to Duffield *et al.* (2009). In the first week postpartum the threshold value of 1.200-1.400 μmol/L of serum BHB indicates the increased health risk and reduced milk production. Elevations in serum BHB during the second week postpartum is associated with increased risk of displaced abomasum and clinical ketosis.

Ospina *et al.* (2010) found similar associations between increased NEFA and BHB concentrations in peripartum period with increased risk of developing of clinical disease which included displaced abomasum, clinical ketosis, metritis and retained placenta. They suggest critical cow-level threshold values as guidelines for the monitoring of cattle in peripartum period. According to their study, concentrations above critical threshold values were associated with increased risk of clinical disease. These threshold values were: 1) 14 to 2 days prepartum NEFA concentrations ≥0.3 mEq/L, 2) 3 to 14 days postpartum NEFA concentrations ≥0.6 mEq/L and BHB concentrations ≥10 mg/dL.

Huzzey *et al.* (2011) investigated association between NEB and health status of the cow during the peripartum period. They reported of strong association between elevated prepartum NEFA serum concentrations with development of postpartum disease within 30

days postpartum. These diseases included retained placenta, displaced abomasum, and subclinical ketosis.

1.6.2. Insulin and insulin-like growth factor

Insulin is the main anabolic hormone that regulates the metabolism of carbohydrates, protein and fat and the body (Sjaastad *et al.*, 2010, P.253). Insulin suppress the ketogenesis, NEFA release from adipose tissue and facilitates the glucose and ketone bodies uptake of peripheral tissues (Youngquist and Threlfall, 2007, P. 358).

IGF-1 is a peptide hormone which mediates the growth-stimulating action of the growth hormone (GH) (Sjaastad *et al.*, 2010, P.235-236). IGF-1 production happens mainly in the liver, but also in the other tissues as well (Sjaastad *et al.*, 2010, P.235-236).
In high producing dairy cows in postpartum period IGF-1 and insulin represent metabolic signals for the resumption of ovarian function (Kawashima *et al.*, 2007). Insulin and IGF-1 regulate the growth of ovulatory follicles and they have essential role in the final stage of follicle development (Spicer *et al.*, 1990; Braw-Tal *et al.*, 2009). More precisely, according to Kawashima *et al.* (2007) plasma concentration of IGF-1 affects the early growth of the ovulatory dominant follicle and insulin is responsible of the maturation and ovulation of the first dominant follicle postpartum at the first follicular wave. Suppression of blood glucose, insulin and IGF-1 concentrations reduce estrogen production by dominant follicle (Butler, 2000). Braw-Tal *et al.* (2009) suggest that abnormal levels of IGF-1 and insulin might lead to follicle dysfunction, resulting in follicular regression or cyst formation.

Decreased IGF-1 concentrations are possibly predictive of inflammation. Valdmann *et al.* (2018) stated in their study that low plasma IGF-1 concentrations antepartum and postpartum were associated with the risk of developing cytological endometritis (CYTO). Cows with IGF-1 concentrations at week -2 prepartum less than 74.6 ng/ml had 3.54 times greater odds of developing CYTO to compared with cows IGF-1 concentrations higher than 74.6 ng/ml.

Cows with IGF-1 concentrations at week +1 postpartum less than 13.2 ng/ml had 4.41 times greater odds developing CYTO than cows with IGF-1 concentration higher than 13.2 ng/ml. Beltman *et al.* (2020) argues that lower IGF-1 concentrations due to poor energy status may inhibit cow's ability to combat bacteria with effective immune response after calving. It may also prolong the uterine involution by delaying the endometrium's repair process. Cows with better energy balance have higher IGF-1 concentrations and this may fasten the rate of uterine involution.

1.7. Enzymes

1.7.1. Aspartate aminotransferase

AST is an enzyme found in the liver, skeletal muscles, and cardiac muscle. It is not tissue specific enzyme and increased levels of AST can be due to injuries to hepatocytes or myocytes (Hoffmann and Solter, 2008. P.356-357).

In cattle AST is used to be considered as muscle- and liver-specific enzyme, but other conditions may also be the cause of increased levels. Kaya *et al*. (2016) reported in their study that cows with endometritis had elevated AST concentrations and these results correlated positively with increase in APPs. Postpartum resumption of luteal activity and fertility was investigated in Estonian Holstein cows by Samarütel *et al.* (2008). Their study showed that cows with delayed resumption of ovarian cyclicity postpartum and prolonged luteal phase had higher serum AST activity 1 to 14 days postpartum compared to normally cycling cows.

1.7.2. Creatine kinase

CK is enzyme found in skeletal and cardiac muscle and it is considered as muscle-specific enzyme. Elevated levels of CK are indicative of muscle injury (Hoffmann and Solter, 2008. P.368).

Sattler and Fürll (2004) studied CK and AST levels in cows as indicators for endometritis and displaced abomasum. In their study significantly lower CK and AST levels ($P < 0.01$) were measured in cows without or with mild endometritis than in cows with moderate or severe endometritis. Sattler and Fürll (2004) suggest that CK can be used as screening parameter for endometritis if elevated CK values due to muscle damage or hypocalcaemia are excluded first.

2. AIM OF THE STUDY

The aim of the study was to establish a set of biomarkers allowing the prediction of cows with risk of developing PPA and to set specific thresholds for the biomarkers.

3. MATERIALS AND METHODS

This study was done as a 6th year veterinary medicine final thesis project. Data set used in this study was previously collected and statistical approach was used to analyse biomarkers related to inflammation, disease, and NEB in dairy cattle. BCS and disease status were also analysed.

3.1. Animals

The collected data set was from 118 multiparous Holstein cows from a single 1200-cow free-stall commercial dairy farm in Estonia. For the experiment, cows were enrolled three weeks before expected calving date and followed to at least day 65 of pregnancy or culling them from the herd. All the calvings took place over the period of 3.3 months and calvings happened in three consecutive series. Cows were fed their respective diets as total mixed ration offered twice daily for ad libitum intake. Cows were milked twice daily.

Estrus was detected with combination of ALPRO (DeLaval, Tumba, Sweden) activity metres, visual observation for standing heat and secondary estrous signs.

3.2. Milk samples

Milk samples for P4 analyses were collected twice weekly. To avoid the effect of time of milk extraction on P4 concentration, samples were collected within 10 minutes following

PM milking on the milking parlor (Waldmann *et al.*, 1999). 10-15 mL milk was withdrawn by stripping and collected in plastic tubes containing potassium dichromate for preservation. Samples were frozen at -20 ºC until P4 analysis.

3.3. Blood samples

Blood sampling was done in three different time points in relation to calving: T1 (-2 weeks prepartum), T2 (+1 week postpartum) and T3 (+3 weeks postpartum). Heparinised tubes (Terumo Europe, Leuven, Belgium) were used in blood collection and plasma was harvested within 30 minutes of sampling following centrifugation at 900 g for 15 minutes. Samples were stored at -20°C for one week at the farm and then at -80°C until analysed.

3.4. Body condition score

BCS was evaluated on a 5-point scale using increments of 0.25 and it was measured for each cow in two different time points: before calving (-2wk prepartum) and after calving (+3wk postpartum). Body condition scoring was performed by the same researcher.

3.5. Disease

Calving related conditions and diseases including twinning, retained placenta, metritis, purulent vaginal discharge, mastitis, and clinical hypocalcaemia were diagnosed and registered by the farm veterinarian. Based on the disease status (no/yes) dummy variable DISEASE was made.

3.6. Analytical procedures

All immunological variables were analysed at The Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Tartu, Estonia. Metabolic variables were analysed at the Department of Animal Health, Welfare and Nutrition, Faculty of Agricultural Sciences, Tjele, Denmark.

3.6.1. Milk progesterone analysis

Concentrations of P4 in milk were measured by an enzyme immunoassay (Waldmann 1993), which was modified by using the second antibody technique. The specificity of the monoclonal antibody and the assay were described previously (Waldmann *et al.,* 1999).

3.6.2. Blood plasma variable analysis

From the blood samples ten different metabolites and hormones were analysed by autoanalyzer or ELISA. IGF-1 concentration in plasma was analysed using human OCTEIA immunoenzymatic plate kits (Immunodiagnostic Systems Ltd, Boldon, Tyne & Wear, UK). Insulin concentration in plasma was analysed using bovine-specific sandwich ELISA (#10–1201–01; Mercodia AB, Uppsala, Sweden). SAA concentration in plasma was analysed using multispecies sandwich ELISA (Phase SAA kit, Tridelta Development Ltd., Maynooth, Ireland). Absorbances were read by a microplate reader Sunrise (Tecan Group Ltd, Männedorf, Switzerland) and calculations were performed by Magellan™ data analysis software (Tecan Group Ltd, Männedorf, Switzerland). The inter- and intra-assay coefficients of variation were <10% for all assays.

Blood plasma samples for all other variables were analysed using appropriate kits and an autoanalyzer, ADVIA 1650 Chemistry System (Siemens Medical Solutions, Tarrytown, NY 10591, USA). All metabolite assay coefficients of variation were low and typically <5%. Blood plasma albumin, AST and CK were determined according to standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1650). Hp was determined chemically due to its ability to bind haemoglobin, Phase ™, Tridelta Developments, Wicklow, Ireland. Cp activity was analysed according to Richterich (1969). NEFAs were determined using the Wako, NEFA C ACS-ACOD assay method. BHB was determined according to Harano *et al.* (1985).

3.7. Data analysis

Cows were grouped into two groups (normal group and PPA group) based on the time of their first ovulation postpartum. PPA cow was defined as a cow experiencing first postpartum milk P4blevel being more than 5 ng/ml at day 50 postpartum or later and normal cow experienced first postpartum milk P4 level being more than 5 ng/ml before day 50 postpartum.

Plasma variables and BCSs in cows with and without PPA were compared using analysis of variance (ANOVA). All variables were logarithm-transformed before ANOVA, because of variables were right-skewed. Optimal thresholds for plasma variable concentrations and BCSs stratified by blood sampling/body condition scoring period for predicting of PPA were obtained by receiver operating characteristic (ROC) curve analysis. Variables with area under curve (AUC) >0.6 and P-value (P) <0.05 were submitted to univariate and multivariate logistic regression models. Forward stepwise algorithm was used in multivariate models. ANOVA and figures were done by using GraphPad Prism 8 Software (8.4.3. Version). Logistic regression and ROC curve analyses were performed with statistical software MedCalc 19.1. (MedCalc, Ostend, Belgium).

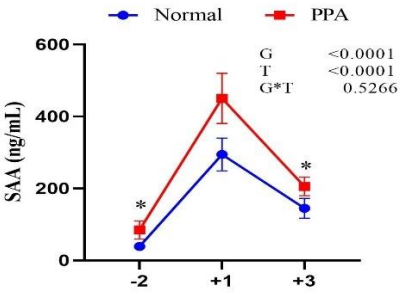
4. RESULTS

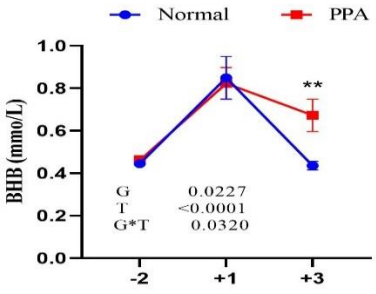
4.1. Prevalence of prolonged postpartum anovulation

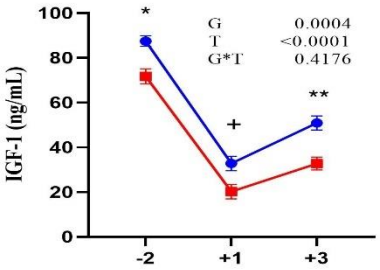
Out of 118 cows 43 (36.44%) cows belonged to PPA group and 75 (63.56%) cows belonged to normal group.

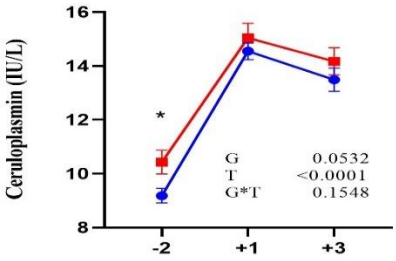
4.2. Circulating blood plasma metabolites, metabolic hormones, and acute phase proteins in cows with or without prolonged postpartum anovulation

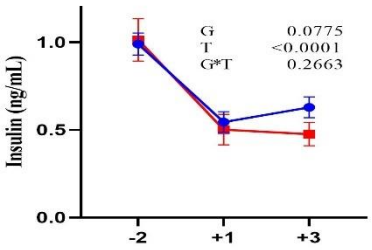
The dynamics of circulating plasma metabolites, metabolic hormones, and APPs in cows with and without PPA at -2wk prepartum, +1wk postpartum and +3wk postpartum is illustrated in Figure 1.

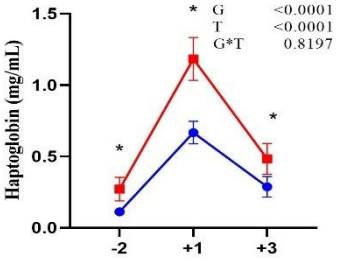

Normal
PPA
SAA (ng/mL)
G <0.0001
T <0.0001
G*T 0.5266
-2
+1
+3

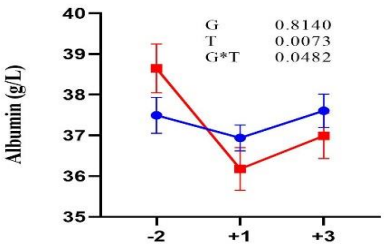

Normal
PPA
BHB (mmol/L)
G 0.0227
T <0.0001
G*T 0.0320
-2
+1
+3

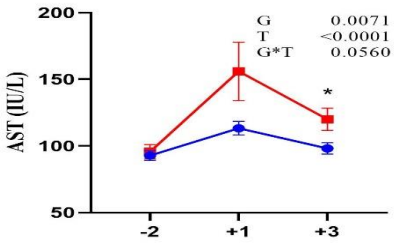

IGF-1 (ng/mL)
G 0.0004
T <0.0001
G*T 0.4176
-2
+1
+3

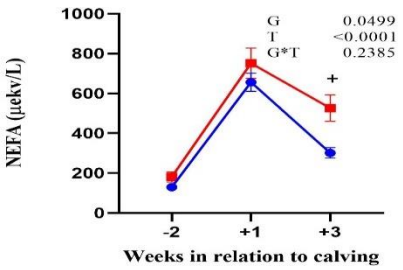

Ceruloplasmin (IU/L)
G 0.0532
T <0.0001
G*T 0.1548
-2
+1
+3


Insulin (ng/mL)
G 0.0775
T <0.0001
G*T 0.2663
-2
+1
+3


Haptoglobin (mg/mL)
G <0.0001
T <0.0001
G*T 0.8197
-2
+1
+3


Albumin (g/L)
G 0.8140
T 0.0073
G*T 0.0482
-2
+1
+3


AST (IU/L)
G 0.0071
T <0.0001
G*T 0.0560
-2
+1
+3


NEFA (µekv/L)
G 0.0499
T <0.0001
G*T 0.2385
-2
+1
+3
Weeks in relation to calving

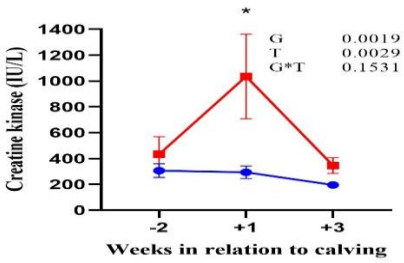

Creatine kinase (IU/L)
G 0.0019
T 0.0029
G*T 0.1531
-2
+1
+3
Weeks in relation to calving

Figure 1. Mean (±se) plasma concentrations of serum amyloid A (SAA), beta-hydroxybutyrate (BHB), insulin-like growth factor-1 (IGF-1), ceruloplasmin (Cp), insulin, haptoglobin (Hp), albumin (Alb), aspartate aminotransferase (AST), non-esterified fatty acids (NEFA), and creatine kinase (CK) at different time points in relation to calving in multiparous Holstein cows with normal resumption of luteal activity and with prolonged postpartum anovulation (PPA). PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later. PPA status group (G), time (T) and group by time interaction (G*T). Symbols indicate means differ *P<0.05; **P< 0.01; ***P<0.001; +P<0.1.

Over the three sampling times cows with PPA had higher plasma concentrations of Hp (P<0.0001) (differing at -2wk (P=0.0163), +1wk (P=0.0237) and +3wk (P=0.0369)), CK (P=0.0019) (differing at +1wk (P=0.0192)), NEFA (P=0.0499) (differing at +3wk (P=0.0557)), AST (P=0.0071) (differing at +3wk (P=0.0476)), SAA (P=0.0001) (differing at -2wk (P=0.0490) and at +3wk (P=0.0203)), BHB (P=0.0227) (differing at +3wk (P=0.0094)), tended to have higher Cp (P=0.0532) (differing at -2wk (P=0.0330)) concentrations, and had lower IGF-1 (P<0.0001) concentrations (differing at -2wk (P=0.0493), +1wk (P=0.0833) and +3wk (P=0.0016)), and tended to have lower insulin concentrations (P=0.0775) in relation to normal group cows. There was no significant group effect regarding albumin concentrations.

A significant (P<0.05) time by group interaction was present in albumin, AST and CK and tended to be in NEFA (P<0.059). Time affected all measured parameters: albumin, CK (P<0.01) and all other variables (P<0.0001), respectively.

4.3. Body condition score in cows with and without prolonged postpartum anovulation

The dynamics of BCS in cows with and without PPA at -2wk prepartum and +3wk postpartum is illustrated in Figure 2. Cows with PPA had lower BCS compared to cows with normal resumption of luteal activity (P=0.0171). BCS was affected by time being lower

(P<0.0001) at +3wk after calving when compared with BCS at -2wk before calving. BCS was lower at +3wk postpartum in cows with PPA when compared to normal cows (P=0.0082).

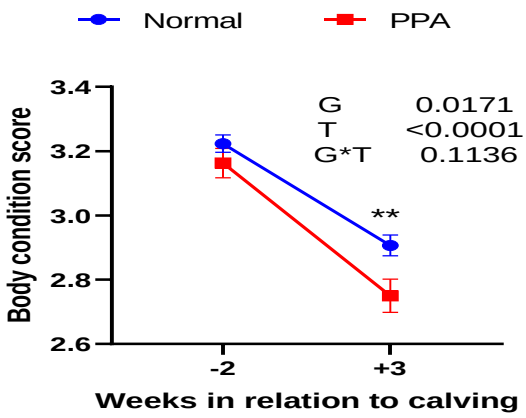


Figure 2. Mean (±se) body condition scores at different time points in relation to calving in multiparous Holstein cows with normal resumption of luteal activity and with prolonged postpartum anovulation (PPA). PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later. PPA status group (G), time (T) and group by time interaction (G*T). Symbols indicate means differ **P<0.01

4.4. Optimal thresholds of plasma variables predicting prolonged postpartum anovulation

Due to the time effect, each blood parameter at each time point was dichotomised for each sampling time by using ROC curve analyses. Optimal thresholds, AUC-s, sensitivities, specificities, and odds ratios for the variables having the areas under the ROC curve >0.6 are shown in Table 1. All dichotomised metabolites and hormones were significant predictors of PPA at least in one sampling time.

Table 1. Optimal thresholds of plasma variable concentrations stratified by sampling period, for predicting prolonged postpartum anovulation (PPA) in multiparous Holstein cows. Sign preceding the threshold indicates the group with the highest occurrence of PPA. Only variables with area under the ROC curve (AUC) >0.6 are shown.

Sampling period	Variable	AUC[1]	Threshold	Se,[2] %	Sp,[3] %	OR[4]	95 %CI[5]	P-value[6]
-2wk before calving	Alb	0.62	>36.90 g/L	88.37	36.00	4.28	1.50–12.15	0.0064
	Cp	0.64	>10.80 IU/L	39.53	86.67	4.25	1.72–10.49	0.0017
	Hp	0.65	>0.07 mg/mL	79.07	42.67	2.81	1.18–6.68	0.0193
	IGF-1	0.69	≤78.04 ng/mL	60.50	68.00	3.14	1.44–6.85	0.0040
	NEFA	0.61	>106 µekv/L	62.80	58.70	2.40	1.11–5.18	0.0263
	SAA	0.64	>5.36 ng/mL	86.00	41.30	4.34	1.63–11.55	0.0032
+1wk after calving	AST	0.63	>116 U/L	51.20	71.10	2.57	1.18–5.59	0.0172
	Hp	0.65	>0.72 mg/mL	62.79	67.11	3.44	1.58–7.52	0.0019
	IGF-1	0.67	≤27.18 ng/mL	79.07	56.58	4.30	1.86–9.96	0.0007
	CK	0.64	>311 IU/L	48.84	76.32	3.08	1.38–6.83	0.0005
+3wk after calving	AST	0.63	>83 IU/L	79.10	44.70	3.06	1.29–7.25	0.0111
	BHB	0.66	>0.60 mmol/L	44.20	89.50	6.73	2.60–17.37	0.0001
	Hp	0.65	>0.19 mg/mL	51.20	78.90	3.93	1.74–8.86	0.0010
	IGF-1	0.70	≤30.80 ng/mL	51.20	81.60	4.23	1.84–9.72	0.0007
	Insulin	0.62	≤0.39 ng/mL	62.80	63.20	2.62	1.22–5.66	0.0140
	CK	0.70	>129 IU/L	81.40	51.30	4.61	1.89–11.23	0.0008
	NEFA	0.64	>431 µekv/L	48.80	82.90	4.63	1.99–10.77	0.0004
	SAA	0.66	>55.56 ng/mL	81.40	53.90	5.13	2.10–12.49	0.0003

[1]Area under the curve, [2]Sensitivity, [3]Specificity, [4]Odds ratio, [5]Cofidence interval, [6]P-value from univariable association of threshold with prolonged postpartum anovulation, Alb=Albumin, Cp=Ceruloplasmin, Hp=Haptoglobin, IGF-1=Insulin-like growth factor 1, NEFA=Non-esterified fatty acids, SAA=Serum amyloid A, AST=Aspartate aminotransferase, CK=Creatin Kinase, BHB=Beta-hydroxy butyrate. PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later

4.4.1. Optimal thresholds of body condition score at +3wk postpartum for predicting prolonged postpartum anovulation

The optimal BCS threshold for predicting PPA, AUC, sensitivity, specificity, and OR for the BCS at +3wk postpartum is shown in Table 2.

Table 2. Optimal threshold of body condition score (BCS) at +3wk postpartum, for predicting prolonged postpartum anovulation (PPA) in multiparous Holstein cows. Sign preceding the threshold indicates the group with the highest occurrence of PPA.

Sampling period	Variable	AUC[1]	Threshold	Se,[2] %	Sp,[3] %	OR[4]	95 %CI[5]	P-value[6]
+3wk after calving	BCS	0.63	<2.75	60.47	76.32	3.54	1.42–8.82	0.0068

[1]Area under the curve, [2]Sensitivity, [3]Specificity, [4]Odds ratio, [5]Cofidence interval, [6]P-value from univariable association of threshold with prolonged postpartum anovulation. PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later

4.5. Multivariate associations of plasma variables for predicting prolonged postpartum anovulation stratified by sampling time

In the multivariate logistic regression models where the blood plasma variables were stratified by sampling time, Alb, Cp, IGF-1, and SAA at -2wk prepartum; Hp, IGF-1 and CK at +1wk postpartum, and BHB, CK and SAA at +3wk postpartum remained significant predictors for PPA in the models (Table 3).

Table 3. Multiple logistic regression models of the association of plasma metabolites measured at -2wk before calving, +1wk and +3wk after calving and body condition score (BCS) at +3wk after calving in multiparous Holstein cows with the risk of subsequent development of prolonged postpartum anovulation (PPA)

Sampling period	Variable	Threshold	Standard Error	OR[1]	95 % CI[2]	P-value[3]
-2wk before calving	Alb	>36.9 g/L	0.62	5.79	1.71–19.64	0.0048
	Cp	>10.8 IU/L	0.56	3.80	1.27–11.38	0.0170
	IGF-1	<78.04 ng/mL	0.45	2.10	0.87–5.04	0.0973
	SAA	>5.36 ng/mL	0.54	2.51	0.86–7.29	0.0916
+1wk after calving	Hp	>0.72 mg/mL	0.44	2.25	0.94–5.36	0.0674
	IGF-1	<27.18 ng/mL	0.47	2.72	1.08–6.85	0.0334
	CK	>311 IU/L	0.44	2.41	1.03–5.66	0.0431
+3wk after calving	BHB	>0.6 mmol/L	0.53	6.51	2.29–18.54	0.0005
	CK	>129 IU/L	0.51	3.28	1.21–8.90	0.0195
	SAA	>55.56 ng/mL	0.51	4.13	1.52–11.22	0.0054
	BCS	<2.75	0.56	4.45	1.48–13.34	0.0078

[1]Odds ratio, [2]Confidence interval, [3]P-value from multivariate association of threshold with prolonged postpartum anovulation, Alb=Albumin, Cp=Ceruloplasmin, Hp=Haptoglobin, IGF-1=Insulin-like growth factor 1, NEFA=Non-esterified fatty acids, SAA=Serum amyloid A, AST=Aspartate aminotransferase, CK=Creatin Kinase, BHB=Beta-hydroxy butyrate. PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later

When all the biomarkers at the three time points (-2wk prepartum, +1wk postpartum, +3wk postpartum) from Table 3 were analysed together using multivariate logistic regression analysis with forward stepwise algorithm, four biomarkers IGF-1, SAA, CK and BHB remained significant in the model (Table 4). The multivariate logistic regression model discriminating cows with and those without PPA generated area under the ROC curve of 0.87 (95% CI = 0.80 - 0.92; P<0.0001).

Table 4. Multiple logistic regression model of association of plasma metabolites and hormones in multiparous Holstein cows with the risk of development of prolonged postpartum anovulation (PPA) when all the biomarkers at three time points (-2wk prepartum, +1wk postpartum, +3wk postpartum) from Table 3 were analysed together

Sampling period	Variable	Threshold	Standard Error	OR[1]	95 % CI[2]	P-value[3]
-2wk before calving	IGF-1	<78.04 ng/mL	0.53	4.17	1.47–11.88	0.0074
	SAA	>5.36ng/mL	0.61	3.77	1.15–12.35	0.0286
+1wk after calving	CK	>311 IU/L	0.53	3.45	1.23–9.64	0.0185
+3wk after calving	BHB	>0.60 mmol/L	0.61	8.15	2.48–26.80	0.0005
	SAA	>55.56 ng/mL	0.56	6.34	2.13–18.93	0.0009

[1]Odds ratio, [2]Confidence interval, [3]P-value from multivariate association of threshold with prolonged postpartum anovulation, Alb=Albumin, Cp=Ceruloplasmin, Hp=Haptoglobin, IGF-1=Insulin-like growth factor 1, NEFA=Non-esterified fatty acids, SAA=Serum amyloid A, AST=Aspartate aminotransferase, CK=Creatin Kinase, BHB=Beta-hydroxy butyrate. PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later.

By including BCS at +3wk postpartum in the multiple logistic regression model presented in Table 4, all metabolites remained significant in the model (Table 5) and BCS was approaching significance. AUC did not change significantly (AUC=0.868 without BCS, AUC=0.875 with BCS) after including BCS in the model. Change in each variable's OR after including BCS ranged from 1 to 15%.

Table 5. Multiple logistic regression model of association of plasma metabolites in multiparous Holstein cows with the risk of development of prolonged postpartum anovulation (PPA) when different time points were analysed together and body condition score (BCS) at +3wk postpartum was included in the model

Sampling period	Variable	Threshold	Standard Error	OR[1]	95 % CI[2]	P-value[3]
-2wk before calving	IGF-1	<78.04 ng/mL	0.55	3.63	1.24–10.64	0.0186
	SAA	>5.36ng/mL	0.60	3.37	1.03–11.04	0.0443
+1wk after calving	CK	>311 IU/L	0.55	4.07	1.40–11.88	0.0101
+3wk after calving	BHB	>0.60 mmol/L	0.62	8.05	2.38–27.28	0.0008
	SAA	>55.56 ng/mL	0.57	6.34	2.08–19.36	0.0012
	BCS	<2.75	0.65	3.16	0.89–11.25	0.0754

[1]Odds ratio, [2]Confidence interval, [3]P-value form multivariate association of threshold with prolonged postpartum anovulation, Alb=Albumin, Cp=Ceruloplasmin, Hp=Haptoglobin, IGF-1=Insulin-like growth factor 1, NEFA=Non-esterified fatty acids, SAA=Serum amyloid A, AST=Aspartate aminotransferase, CK=Creatin Kinase, BHB=Beta-hydroxy butyrate. PPA was defined as a cow experiencing first postpartum milk progesterone rise >5 ng/ml at day 50 postpartum or later

Disease status was not a significant variable in predicting PPA in univariate logistic regression analysis (P=0.17).

When disease status was included as a covariate into the multiple logistic regression model, it did not become significant variable (P=0.75) and was omitted from the final model.

5. DISCUSSION

The impairing effect of NEB and inflammation on postpartum resumption of ovarian activity in dairy cattle is well known matter and many dairy herds face the problem of PPA in cows. Prevalence of PPA in dairy cattle herds varies, but alarm level of ≥21% of PPA in dairy herds is suggested by Dubuc *et al*. (2017). By monitoring biomarkers associated with inflammation and inflammatory diseases like SAA, Hp, Cp, albumin, and biomarkers associated with NEB like NEFA, BHB and IGF-1, could give a chance of decreasing the number of cows experiencing PPA. In this study we established a set of biomarkers which would potentially help in prediction of PPA in dairy cows.

The prevalence of PPA in our study was 36.4% and this is in line with the 35.2% reported by Dubuc *et al.* (2017) in multiparous cows. Lower PPA prevalence is reported by Moreira *et al*. (2001), Walsh *et al*. (2007), Santos *et al*. (2009) and Dubuc *et al*. (2012) where it varied between 19% to 26.2%, but these study populations consisted of both primiparous and multiparous cows. Cows of third parity or greater were shown to have lower probability of early ovulation than first and second parity cows (Dubuc *et al.*, 2012), but Plozza *et al*. (2016) and Santos *et al*. (2009) argue that primiparous cows are more likely to express PPA period than multiparous cows. According to Dubuc *et al.* (2017) the variation in the prevalence of PPA results can be explained by the different levels of herd management and nutrition status of individual cows at farm level.

Postpartum resumption of ovarian cyclicity is dependent on LH pulse frequency and this process is negatively affected by NEB (Crowe *et al.* 2014). Plasma BHB concentrations reflect NEB and dairy cows with severe NEB have higher circulating BHB concentrations compared to cows with mild NEB (Llewellyn *et al.*, 2007). IGF-1 have essential role in the follicular growth before ovulation and abnormal levels of IGF-1 might lead to follicle

dysfunction and be a cause of PPA (Braw-Tal *et al.,* 2009). Cows experiencing NEB have lower plasma IGF-1 concentration than cows with positive energy balance (Spicer *et al.,* 1990).

Incidence of inflammatory diseases and inflammations in the herd affects the prevalence of PPA. Common inflammatory diseases in dairy cows are clinical and subclinical endometritis. Endometritis delays the onset of postpartum ovarian cyclicity (Sheldon *et al*., 2009). During inflammation bacteria, their products, and endotoxins can impair the ovarian function via activation of PRR e.g., TLR (Gilbert, 2019). These receptors respond to pathogen-associated molecular patterns (PAMPs) found in the cell walls of bacteria. LPSs are typical PAMPs found in gram-positive and gram-negative bacteria (Draing *et al*., 2008; Gilbert, 2019). Concentrations of acute phase proteins reflect the magnitude of endotoxin LPS exposure (Jacobsen et al., 2004). Inflammation does not need to be uterine related to be able to impair or affect the ovarian function. Endotoxins like LPS can directly reach ovaries via general circulation from mucosal tissues of distant sites (Draing *et al*., 2008; Gilbert, 2019). For cattle typical inflammatory conditions where endotoxins are released and can reach general blood circulation are mastitis, retained placenta, uterine infections (metritis, endometritis) and laminitis (Draing *et al.* 2008). LPS can suppress oestradiol production in dominant follicles and recruited follicles (Herath *et al*., 2007). Another way for endotoxins to reach general circulation is via gastrointestinal tract. Khafipour *et al.* (2009) reported strong association between rumen and peripheral LPS concentrations during subacute rumen acidosis (SARA), whereas Plazier *et al.* (2012) reported inconsistency of peripheral LPS concentrations during SARA.

Findings of this study support the previous research stating that NEB and inflammation have impairing effect on resumption of postpartum ovarian cyclicity. In the univariate logistic regression models several variables mirroring low energy and inflammation were related to PPA (Table 1) in different time points (2wk prepartum, +1wk postpartum, +3wk postpartum). Because the measured variable's threshold values changed in time, each time point was analyzed separately. After submitting the significant variables into multivariate logistic regression analysis, variables with strongest connection to PPA stayed in the model

(Table 3). In general, there is very little published studies on biomarkers related to PPA and their specific threshold values, whereas there is more information on biomarkers predicting diseases, inflammation, and NEB.

In the multivariable analysis cows with elevated plasma albumin, SAA and Cp and lower IGF-1 concentrations at -2wk prepartum were at higher risk of PPA. Albumin, SAA, and Cp are indicative for inflammation and IGF-1 for energy balance. Albumin is a negative APP, and plasma albumin concentration decrease normally during inflammation (Eckersall, 2008. P.132-135). In multiparous cows, low albumin concentrations at -2wk prepartum were predictive for metritis and retained placenta (Ruprechter *et al.,* 2018). However, in our study the PPA group cows had higher plasma albumin concentrations at -2wk prepartum compared to normal group cows. Plasma albumin concentrations at -2wk prepartum correlated significantly with NEFA concentrations measured at +1wk postpartum ($r=0.2242$; $P=0.0147$) and BHB concentrations measured at +3wk postpartum ($r=0.2872$; $P=0.0016$). Such relationship indicates that cows with higher albumin concentration before calving may be in deeper NEB after calving. This would explain why high prepartum albumin was related to PPA in our study. SAA and Cp are positive APPs, and their plasma concentrations increase at inflammation (Eckersall, 2008. P. 137-138; Cerone *et al.*, 2000). Metabolic hormone IGF-1 has essential role in follicular growth before ovulation and abnormal levels can result follicular dysfunction which can lead to PPA (Braw-Tal *et al.,* 2009).

In the multivariable analysis at +1wk postpartum elevated plasma Hp, CK and lower IGF-1 concentrations indicated the risk of PPA. Dubuc *et al.* (2012) showed that elevated Hp concentration (≥0.3 g/L) during the 15-21 days postpartum was associated with a longer period of postpartum anovulation. The threshold ≥0.3 g/L reported by Dubuc *et al.* (2012) is not directly comparable to our Hp thresholds due to different sampling time. However, the threshold 0.3 g/L is between 0.72 and 0.19 mg/mL established in the present study for +1wk and +3wk postpartum, respectively. The rapid change in Hp concentrations in time suggest that the time-windows for measuring Hp should be narrow. Hp levels were significantly higher in cows with endometritis than in healthy cows and the concentration correlates with the severity of endometritis (Kaya *et al.,* 2016). There are indications that Hp concentrations

in the blood plasma could be used as predictive biomarker for inflammation. According to Huzzey *et al.* (2009) acute phase inflammatory response precedes clinical metritis. Their study showed that postpartum cows 3 DIM with Hp concentration ≥ 1 g/L were 6.7 times more likely to develop severe or mild metritis. Elevated levels of CK are indicative of muscle injury (Hoffmann and Solter, 2008. P.368). In the present study higher plasma CK concentrations at +1wk postpartum were indicative for PPA. Higher CK concentrations may have resulted from myometrial damage and inflammation caused by parturition. Sattler and Fürll, (2004) have suggested CK, as screening parameter for endometritis if elevated CK values due to muscle damage or hypocalcaemia are excluded first. Samarütel *et al.* (2008) found connection between cows with delayed resumption of ovarian cyclicity having higher serum AST activity 1 to 14 days postpartum compared to normally cycling cows. In line with the findings of Samarütel *et al.* (2008) higher AST activity at +1wk and +3wk postpartum was associated with PPA in univariate analyses, but in the multivariate analyses AST did not remain significant variable in the model. Lower IGF-1 concentrations indicate poor energy status. Low level of plasma IGF-1 concentration is related to NEB and Llewellyn *et al.* (2007) reported significantly lower levels of IGF-1 in cows with severe NEB compared to cows with mild NEB.

At +3wk postpartum elevated SAA, CK and BHB concentrations indicated the risk of PPA. Blood NEFA and BHB concentrations are used as markers of NEB in dairy cattle and the level of NEFA and BHB concentrations are related to the severity of NEB (Llewellyn *et al.*, 2007). Elevated blood plasma BHB concentrations have been shown to be associated with postpartum diseases. Ospina *et al.* (2010) set threshold value for BHB ≥10 mg/dL in period of 3 to 14 days postpartum. Blood concentrations above this threshold was associated with increased risk of displaced abomasum, clinical ketosis, metritis and retained placenta. Jeong *et al.* (2015) reported higher BHB concentrations in non-cycling cows (serum P4 concentration ≤1ng/mL ≥6 weeks postpartum) immediately after calving compared to normally cycling cows. This is different form our result, where BHB concentration was higher in PPA group cows at +3wk postpartum. Role of elevated SAA and CK concentrations in relation of developing PPA are the same as mentioned earlier.

When analyzing all three time points (-2wk, +1wk and +3wk in relation to calving) together, biomarkers that remained significant in the multivariate logistic model were at -2wk prepartum IGF-1 (threshold < 78.04ng/mL) and SAA (threshold > 5.36 ng/mL), at +1wk postpartum CK (threshold > 311 mg/dL) and at +3wk postpartum SAA (threshold > 55.56 ng/mL) and BHB (threshold >0.60 mmol/L). These biomarkers reflect the inflammatory, nutritional and muscle injury status of a dairy cow. Accuracy (AUC 0.86) of the final model is good reflected to the traditional academic point system; excellent=0.90-1.0, good=0.80-0.90, fair=0.70-0.80, poor=0.60-0.70, fail=0.50-0.60 (Murphy *et al.* 2005-2020). Very interesting finding in the final model is the role of SAA being significant biomarker for PPA already before calving and then again at +3wk after calving. Only biomarker left in the model at time point +1wk postpartum is the CK, which suggest that muscle injuries and infections during parturition increase the risk of PPA. Thirdly, there is change of the energy balance biomarkers in time; IGF-1 is significant before calving, but at +3wk after calving it is BHB.

I was not able find related studies on biomarkers in prediction of PPA or related threshold values. Ruprechter *et al.* (2018) identified that multiparous cows with at least two disease events (retained placenta, metritis, mastitis, hypocalcemia, ketosis) had higher BHB concentrations at +3wk when compared with healthy cows. This correlates with our BHB result at +3wk postpartum, but our study predicted PPA, not diseases. Stevenson *et al.* (2020) reported that increased levels of BHB and Hp postpartum were found in cows diagnosed with peripartum disease and that disease delayed first postpartum anovulation. Opsomer *et al.* (2000) showed that abnormal calving and diseases such as clinical mastitis, severe lameness, pneumonia, and clinical ketosis during the first moth after calving are risk factors for delayed postpartum resumption of ovarian activity. As NEB and inflammation negatively affects the resumption of postpartum ovarian activity, detecting such conditions as early as possible gives a chance to improve reproductive efficiency in dairy cows.

In the present study the number of the animals was small, and that is why all the disease conditions were analyzed together as one variable. Perhaps that is the reason why disease status of a cow did not become significant variable in predicting PPA. However, the

significant metabolites in the multivariate logistic regression model describe well the disease status and APPs describe the severity of the inflammation.

BCS reflects the nutritional status of the cow. According to Pryce *et al*. (2001), Santos *et al*. (2009) and Plozza *et al.* (2016) cow's decreasing BCS in early postpartum period has negative effect on resumption of ovarian function and reproductive success. Our results showed that the optimum threshold for BCS at +3wk postpartum was 2.75 and cows with BCS < 2.75 had 3.54 times greater odds developing PPA (P=0.0068). Maintaining the cow at the optimal BCS around the periparturient period decreases the risk of developing PPA.

Incorporation of BCS at +3wk postpartum into the final model did not improve the model. AUC suggest that final model predicts PPA well without BCS. The AUCs were 0.87 and 0.63 for the metabolites based and only BCS based models, respectively. Measuring BCS at +3wk postpartum can be used as tool to predict PPA in dairy cows, but the predictability is not as accurate as in the metabolite based final model. There was small (range from 1 to 15%) change in plasma variable's ORs, but none of the ORs changed >15% after including BCS. This indicates that inclusion of BCS into the final model had very small effect on the plasma variables in the model.

Based on our study results, the prevalence of PPA in dairy herds can be diminished by preventing severe NEB and identifying and managing inflammatory diseases as early as possible. Sampling cows at -2wk prepartum and screening the ones with bad set of biomarkers helps to identify animals with high risk for delayed resumption of postpartum ovarian cyclicity. Early screening enables to make e.g., feeding changes to avoid NEB. Separating these high risk animals from the herd makes monitoring easier and helps diagnosing inflammatory conditions earlier and treat them effectively. Resampling high risk animals in every two weeks helps to follow up the prevalence change of PPA in the herd. As this study showed, time has significant effect on the variables, and they change quick in time as do their thresholds. Observing and maintaining the cows at the optimal BCS level before and after calving is also key element on supporting their postpartum resumption of ovarian activity.

5.1. Future research

More research is needed on this topic before results can be put into practice. Limitations in this study were small study group size (n=118), all cows were multiparous and from the same farm. Future research should include larger groups sizes, primiparous cows, different farms, environments, seasons, and dairy cows breeds. Disease status of the cow was not contributing to the risk of developing PPA in present study. With larger study groups size, it would be possible to concentrate on separate disease conditions and to find out will disease status become risk factor for PPA. It would be interesting to compare which is better predictor of PPA, disease status or metabolites or are both needed for best prediction of PPA.

6. CONCLUSIONS

This study gave a set of biomarkers for prediction of PPA in multiparous dairy cows. Set of biomarkers were at -2wk prepartum IGF-1 and SAA, at +1wk postpartum CK and at +3wk postpartum BHB and SAA. These biomarkers are related to NEB (IGF-1, BHB), inflammation (SAA) and muscle damage (CK). Time significantly affected the biomarkers concentrations and their threshold values; therefore, the sampling time window should be kept narrow ≤ 1wk when blood samples are collected for metabolic evaluation. The final model based only on blood metabolites had good accuracy in predicting PPA. Inclusion of BCS did not improve the AUC of the final model. BCS at +3wk postpartum can be used for predicting PPA, but with significantly lower accuracy when compared with the biomarker-based model.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor professor Andres Valdmann for presenting me this topic and giving me the opportunity to write my final year thesis about it. I highly appreciate his support, guidance, and endless patience throughout the whole final thesis process. I would also like to thank professor Tanel Kaart for his contribution with statistical procedures.

APPENDIXES

Appendix 5. Non-exclusive licence for depositing the final thesis and opening it for the public and the supervisor's (supervisors') confirmation for allowing the thesis for the defence

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